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(54) Title: HUMAN MONOCYTE/MACROPHAGE DERIVED METALLOPROTEINASE INHIBITOR

(57) Abstract

The present invention provides nucleotide sequences that identify TIMP-3 produced by human macrophages and/or monocytes. The present invention also provides for nucleotide sequences encoding TIMP-3, expression vectors for the production of purified TIMP-3, antibodies capable of specifically binding to TIMP-3, hybridization probes for the detection of TIMP-3 encoding nucleotide sequences, and genetically engineered host cells for the expression of TIMP-3.

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Human Monocyte/Macrophage Derived M talloproteinase Inhibitor

FIELD OF THE INVENTION

The present invention is in the field of molecular biology; more particularly, the present invention describes a protein produced by human monocyte/macrophage cells and nucleic acid sequences encoding this protein. Specifically the present invention describes a new metalloproteinase inhibitor, TIMP-3, which is produced by monocyte/macrophage cells and is useful for regulating tissue destruction by metalloproteinases.

BACKGROUND OF THE INVENTION

Monocytes/macrophages play a central role in host defense and the inflammatory process. Monocyte functions include phagocytosis and killing of pathogenic microorganisms, processing of antigens for "presentation" to T cells, antibody dependent and independent cytotoxicity, and secretion of numerous cytokines that play a key role in the interaction of monocyte/macrophages with a variety of lymphocytes and other cells to generate an inflammatory response. Therefore, characterizing the proteins produced by monocytes/macrophages, identifying the key proteins and developing immunoassays for the presence of monocytes in inflammation represents a significant advance in monocyte therapeutic and diagnostic approaches.

Monocytes develop from monoblasts through promonocytes in the bone marrow and eventually join a pool of circulating mature monocytes which have a half-life in the blood stream of approximately three days. Roughly 75% of the circulating monocyte pool marginates along blood vessel walls. Monocytes randomly migrate to the tissues to become antigen presenting cells or phagocytic macrophages. The phagocytic macrophages include alveolar macrophages of the lung, bone marrow macrophages, and Kupffer cells of the liver. The antigen presenting or dendritic cells include the interdigitating

reticular cells and follicular dendritic c lls of the lymph nodes and the Langerhans cells of the skin. Thus monocytes and macrophages play a key role in host defense both in the blood and in tissues. Characterizing a large number of the proteins synthesized by the monocyte/macrophage represents a major breakthrough in the study and development of therapeutic and diagnostic approaches to monocyte-mediated inflammatory events and provides a commercially useful library of monocyte/macrophage-derived polypeptides and cDNAs.

Macrophages can be distinguished from monocytes by several different cellular characteristics. Precursor monocytes are rich in azurophilic peroxidase-containing cytoplasmic granules and contain large amounts of myeloperoxidase. Macrophages have more cell surface receptors by which they can "sense" their environment. These include receptors for: immunoglobulin and complement, growth factors, lipoproteins, peptides and polysaccharides. Binding of ligands to these receptors triggers macrophage proliferation, chemotaxis, secretion and phagocytosis--the process of internalization of material outside of the cell. This type of activation enables these cells to intensify their killing of intracellular microorganisms. In addition to the antimicrobial function of macrophages, these cells perform the functions of immunoregulation, inflammation, wound healing and the selective removal of tumor cells, senescent cells or those cells which are metabolically defective. Macrophages also have the capability to function as secretory and regulatory cells, producing many different substances involved in the inflammatory response. Macrophages play an important role in pathogenesis and disease states, and also in embryonic development and postnatal brain remodeling. Inherited diseases involving macrophages and monocytes include: 1) storage diseases manifesting a deficiency of degradative enzymes, 2) malignant osteoporosis, 3) chronic granulomatous disease, and 4) familial chronic mucocutaneous candidiasis. Some of these diseases may be able to be corrected by bone marrow transplant or retroviral mediated gene transfer. In

addition, macrophage disorders can cause d fective handling of bacteria upon infection, including improper bactericidal killing, bowel damage, arthritis, and toxic shock syndrome. Physical and chemical agents, including trauma, smoking, UV irradiation, asbestos exposure and steroids can block proper functioning of macrophages and monocytes. Macrophages which accumulate cholesterol from blood lipoproteins become the foam cells of human atherosclerotic lesions. Finally, autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis cause abnormal function of monocytes.

THP-1 CELLS

THP-1 is a human leukemic cell line with distinct monocytic characteristics. This cell line was derived from the blood of a 1 year old boy with acute monocytic leukemia (Int. J. Cancer: 171-176 (80)). The following cytological and cytochemical criteria were used to determine the monocytic nature of the cell line: 1) the presence of a-naphthyl butyrate esterase activity which could be inhibited by NaF (sodium fluoride), 2) the production of lysozyme, 3) the phagocytosis of latex particles and sensitized SRBC (sheep red blood cells), and 4) the ability of mitomycin C-treated THP-1 cells to activate T-lymphocytes following ConA (concanavalin A) treatment. Morphologically, the cytoplasm contained small azurophilic granules and the nucleus was indented and irregularly shaped with deep folds. The cell line had Fc and C3b receptors, probably functioning in phagocytosis.

If one looks at the continuum of normal hematopoietic differentiation, leukemia could be considered to represent an arrest in normal differentiation, and leukemic cell lines would reflect the "block" in differentiation intrinsic to the cells from which the line was derived. Many human myeloid and myelomonocytic cell lines retain some ability to differentiate into more mature phenotypes in response to various stimuli including growth factors, lymphokines, cytokines, vitamin D derivatives, and tumor promoters. THP-1 cells treated with the tumor promoter 12-O-tetradecanoyl-phorbol-13 acetate (TPA)

will stop proliferating and differentiate into macrophage-like cells which mimic native monocyte-derived macrophages in several respects. Morphologically, as the cells change shape the nucleus becomes more irregular and additional phagocytic vacuoles appear in the cytoplasm. The differentiated THP-1 cells also exhibit an increased adherence to tissue culture plastic.

In addition to physical changes, induced THP-1 cell lines also exhibit characteristic changes in gene expression. Proto-oncogenes are involved in the regulation of cellular proliferation and differentiation via various signal transduction pathways. They have been shown to undergo altered levels of expression following the differentiation of monocytes or myeloid cell lines into macrophages and can be used to monitor the maturation of myelomonocytic leukemia cells and the differentiation of THP-1 cells exposed to various inducing agents including TPA. C-fos and c-jun, transcriptional activators, are coinduced upon differentiation, c-myc remains unchanged, and c-myb is down-regulated during the differentiation of these cells (Experientia 47: 22-30 (91)).

As myeloid precursor cells differentiate into mature cells, a change in membrane molecules is observed associated with the acquisition of both phagocytic and microbicidal capabilities involved in the host defense response. Following TPA induction of THP-1 cells, an increase in density of the complement C3b receptor and decrease in both FcR and CD4 is observed, the later being an adhesion molecule. These changes are also associated with the maturation of peripheral blood monocytes into macrophages.

Another characteristic of macrophages is the ability to respond to physiological stimuli by the secretion of a wide range of physiologically active molecules, including peptide hormones, cytokines, enzymes, and reactive oxygen species. Two proteins involved in lipid metabolism and also associated with atherosclerotic lesions are lipoprotein lipase (LPL) and apolipoprotein E (apo E) both produced by THP-1 cells and

macrophages. LPL is responsible for the hydr lysis of the core triglycerides in triglyceride-rich lipoproteins to glycerol and free fatty acids and is expressed in THP-1 cells following TPA-induced differentiation. Apo E, an important component of several plasma lipoproteins, is associated with the redistribution of cholesterol between cells and tissues and also increases following differentiation.

Once monocytes have matured into macrophages, distinct patterns of cytokine production are associated with the "activation" of these cells. Discrete stimuli cause these activated macrophages to secrete several proinflammatory cytokines, including IL-1B and TNF, both of which elicit potent biological effects contributing to the inflammatory joint disease and bone destruction that characterize rheumatoid arthritis. Both TPA and lipopolysaccharide (LPS or "endotoxin") induce expression of IL-1B in THP-1 cells, with TPA "priming" the macrophages for enhanced secretion (Agents and Actions 27: 271-273 (89)). IL-1 antagonists, secreted by PMA-differentiated THP-1 cells, have been shown to inhibit in vitro activities of IL-1 and therefore could be useful in the development of anti-inflammatory molecules for chronic inflammatory disease (J. Biol. Chem. 265: 14505-14511 (85)).

MATRIX METALLOPROTEASES (MMPs) AND INHIBITORS

The protein molecule of this invention (TIMP-3) is a novel member of a class of protease inhibitors known as tissue inhibitors of metalloproteinases (TIMPs). As is implied by their name, the key feature of these proteins lies in their ability to inhibit one or more of the metalloproteinases, a class of proteolytic enzymes that require a Zn^{++} cation for activity. Insofar as the inhibitor of this invention may inhibit any one or several of the metalloproteinases, a consideration of the function and importance of the metalloproteinases is germane to understanding the potential utility of the inhibitor.

The metalloproteinases are a multigene family which participate in a variety of biologically important processes by virtue of their ability to degrade many components of extracellular matrix (ECM). Below is a table of the reported human metalloproteinases and a partial listing of known ECM substrates.

MMP-1	Interstitial collagenase	Collagens I, II, III, VII, X
MMP-8	Neutrophil collagenase	Collagens I, II, III
MMP-2	72 kDa gelatinase	Collagens IV, V, VII, X, Fibronectin, Elastin
MMP-9	92 kDa gelatinase	Collagens III, IV, V, IX
MMP-3	Stromelysin	Collagens I, II, III, Fibronectin, Laminin, Proteoglycan link protein
MMP-10	Stromelysin-2	Fibronectin, Collagens III, IV, V
MMP-7	Uterine Metalloproteinase	Fibronectin, Proteoglycan

Metalloproteinases also proteolytically inactivate several members of a class of serine protease inhibitors (serpins), which in turn function to regulate the activity of many potent serine proteases. By destroying the serpins, the MMPs indirectly bring about the destruction of a variety of biologically important molecules by serine proteases. Thus, controlling the activity of the MMPs has indirect effects on activity of a range of other proteases.
As can be seen from the substrates listed in the table, MMPs are capable of degrading many of the key structural component of tissues. This ability is utilized in normal processes where destruction of existing tissues is necessary, such as embryogenesis and tissue remodeling. Metalloproteinases have also been implicated in processes where their activity is more specifically directed, such as in the movement of cells through tissues.

In addition, the MMPs play an important role in a number of pathological processes. In rheumatoid and other forms of arthritis, proteolysis of ECM components by MMPs is a major cause of synovial tissue destruction seen in these diseases (reviewed in Curr. Opin. Rheumatol. (1992) 4, 348-54.

M talloproteinases also participat is in tumor metastasis. In order to reach secondary sites, primary tumor cells must first gain access to the circulation and later reenter the tissues. Both involve moving through tissues as well as the endothelial basement membrane. The degradative action of metalloproteases on these structures is critical for the migration of tumor cells (reviewed in Mignatti, P. and Rifkin, D.B. (1993) Physiol. Rev. 73, 161-95).

MMPs have been implicated in periodontal disease. Inflammation of the periodontium leads to connective tissue degradation and eventually to tooth loss. Tissue degradation is largely mediated by neutrophils attracted to the inflammatory site, and secretion of neutrophil MMPs is believed to play a major role in tissue destruction (reviewed in Sodek, J. and overall, C.M. (1992) Matrix Suppl 1, 352-62).

Metalloproteinases participate in bone resorption process. Collagenase produced by osteoblast cells degrades collagen overlying the surface of the bone and affords osteoclasts access to underlying mineralized bone. The osteoclasts in turn utilize MMPs as well as other proteases in bone resorption. This bone catabolism affects the overall balance of deposition/resorption and resulting imbalance can lead to demineralization of the bone and osteoporosis (Everts, V. et al. (1992) J. Cell Physiol. 150, 221-231; Vaes, G. et al. (1992) Matrix, Suppl. 1, 383-388).

There is evidence for MMP involvement in corneal ulcer formation following alkali burns and bacterial inflammation (Wentworth, J.S. et al. (1992) 33, 2174-9; Burns, F.R. et al. (1992) Matrix, Suppl 1, 317-318. Inhibition of MMPs may have a therapeutic effect in prevention of corneal ulceration resulting from a broad variety of insults.

The involvements of MMPs in this wide range of pathological conditions suggest that inhibitors of these enzymes would have broad therapeutic potential. As natural inhibitors of the MMPs, TIMPs should be medically useful for treatment of pathological conditions involving MMPs including but not limited to those discussed above.

BACKGROUND ART

There are two human TIMPs previously characterized on the sequ nce level, TIMP-1 (Docherty, A.J.P. et al (1985) Nature 318, 66-69 and TIMP-2 (Boone, T.C. et al. (1990) Proc. Nat. Acad Sci. 87, 2800-2804; Stetler-Stevenson, W.G. et al (1990) J. Biol. Chem. 265, 13933-38). The proteins are classified in the TIMP family based on their structural similarity to each other as well as their mutual ability to inhibit metalloproteinases. Although both human TIMPs inhibit a range of metalloproteinases, their specificities do differ. TIMP-2 is a more effective inhibitor of both 72kDa gelatinase (MMP-2) and 92kDa gelatinase (MMP-9), while TIMP-1 is the better inhibitor of MMP-1, interstitial collagenase (Howard, E.W. et al. (1991) J. Biol. Chem. 266, 13070-75). In addition, there have been other reports of inhibitors of metalloproteinases (IMPs) with physical characteristics different from those of TIMP-1 or TIMP-2. In some cases these activities result from alternate forms of the known TIMPs. For instance, a recent report describes one IMP present in the conditioned media of a human bladder carcinoma to be a partially glycosylated form of TIMP-1 and another to be a partially processed/degraded form of TIMP-2 (Miyazaki, K. et al. (1993) J. Biol. Chem. 268, 14387-93). There are additional reports that describe sources and characteristics of IMP activity, but the gene products associated with these activities have not been delineated (Apodaca, G. et al. (1990) Cancer Research, 50, 2322-29). Thus, TIMP-1 and TIMP-2 are the only human TIMPs that have been fully characterized and cloned. It is impossible to know which of the activities described are due to novel members of the TIMP family or how many members of the TIMP family are encoded in the human genome.

Additional TIMPs have been cloned from other species including bovine TIMP-1 (Freudenstein et al. (1990) Biochem. Biophys. Res. Comm. 171, 250-256) and TIMP-2 (Boone, T.C. et al. (1990) Proc. Nat. Acad Sci. 87, 2800-2804), murine TIMP-1 (Gewert, D.R. et al. (1987) EMBO J. 6,651-657), rabbit TIMP-1 (Horowitz, S. et al. (1989) J. Biol. Chem. 264, 7092-7095) and

chicken TIMP-3 (Pavloff, N. et al. (1992) J. Bi l. Chem. 267, 17321-6). Chicken TIMP-3 is more closely homologous to TIMP-2 than TIMP-1 of other species. Cloned and fully characterized homologs of TIMP-1 and TIMP-2 from chickens have not been reported. The protein of this invention is closely related to the TIMP family. Homology of this protein to human TIMP-1 and TIMP-2 is shown in Table 1. By sequence homology, the protein of this invention is clearly a new member of the human TIMP family. We therefore call this molecule human TIMP-3. Of the TIMP molecules reported so far, human TIMP-3 is most closely related to the chicken TIMP-3. The sequence alignment between these two molecules is shown in Table 1.

SUMMARY OF THE INVENTION

The subject invention provides a nucleotide sequence that uniquely identifies a novel human monocyte/macrophage derived protein. We designate this new gene "tissue inhibitor of metalloproteinases-3" or TIMP-3. Aspects of the subject invention include: purified TIMP-3; purified nucleotide sequences encoding all or a portion of said TIMP-3; recombinant host cells or organisms for the production of TIMP-3; hybridization probes for detecting nucleotide sequences encoding TIMP-3; hybridization probes for detecting nucleotide sequences encoding TIMP-3; hybridization probes useful for regulating TIMP-3 gene transcription or translation; antibodies capable of specifically binding to TIMP-3; methods of detecting TIMP-3 with TIMP-3-specific antibodies, particularly in biological samples; methods of isolating recombinant or naturally-occurring TIMP-3 using TIMP-3-specific antibodies; methods of treating samples with TIMP-3 to inhibit proteolytic activity in the sample; pharmaceutical compositions comprising TIMP-3 in an acceptable excipient and methods of treating diseases in which matrix metalloproteases play a role.

DESCRIPTION OF THE TABLE

Table 1 shows an amino acid homology analysis of chicken TIMP-3 and human TIMP-1, TIMP-2 and TIMP-3.

DESCRIPTION OF THE FIGURES

Figure 1 shows and amino acid content graph of human TIMP-1.

Figure 2 shows and amino acid content graph of human TIMP-2.

Figure 3 shows and amino acid content graph of human TIMP-3.

Figure 4 shows an isoelectric point curve of human TIMP-1.

Figure 5 shows an isoelectric point curve of human TIMP-2.

Figure 6 shows an isoelectric point curve of human TIMP-3.

Figure 7 shows a hydrophobicity profile of human TIMP-1.

Figure 8 shows a hydrophobicity profile of human TIMP-2.

Figure 9 shows a hydrophobicity profile of human TIMP-3.

Figures 10 A-C show RNA "Northern" blots of polyadenylated RNA isolated from various human tissues using a TIMP-3 probe.

Figure 11 shows a Southern blot of human chromosomal DNA digested with various restriction enzymes, and hybridized with a TIMP-3 probe.

Figure 12 shows a Southern blot of Eco RI-digested chromosomal DNA taken a variety of mammalian somatic cell hybrids that has been hydribized with a TIMP-3 probe.

Figure 13 shows a "zoo" blot of Eco RI-digested chromosomal DNA from a variety of mammalian sources that has been hybridized with a TIMP-3 probe

DETAILED DESCRIPTION OF THE INVENTION

Definitions

As used herein the term "TIMP-3" refers to endogenously encoded, i.e., a naturally occurring, polypeptide expressed by

human THP-1 cells activated with endot xin having the amino acid sequence backbone described in Sequence I.D. No 1 in the sequence listing and to allelic variants, derivatives, fragments, or recombinant forms that retain the biological characteristics of the naturally occurring human TIMP-3, i.e., metalloproteinase inhibitory activity. The term "naturally occurring TIMP-3" refers to TIMP-3 with a primary amino acid sequence that is the same as the primary amino acid sequence of a TIMP-3 produced by a non-genetically engineered human cell in vitro or in vivo.

The term "TIMP-3 derivative" is defined to include polypeptides possessing TIMP-3 biological activity and/or TIMP-3 immunological activity. The terms "biological activity" (or "biological characteristics") of a protein refers to the structural or biochemical function of the protein in the normal biological processes of the organism in which the protein naturally occurs. Examples of biological characteristics of TIMP-3 include both its structure and/or conformation, which can be determined by specific antigenicity or immunogenicity, and the ability of the TIMP-3 to mediate inflammatory responses by the regulation of metalloproteinases in vivo. The term "biological activity" of TIMP-3 includes the range of biological characteristics associated with the native form of TIMP-3.

By TIMP-3 "immunological activity," it is intended that a polypeptide with TIMP-3 immunological activity can specifically bind with antibodies specific for TIMP-3, or can, upon injection into appropriate animals (with suitable adjuvants), be used to induce an immune response specific for TIMP-3. Unless indicated otherwise, the term "TIMP-3 derivative" means TIMP-3 derivatives with biological activity and TIMP-3 immunological activity (i.e. are immunologically cross-reactive with antibodies specific for TIMP-3).

Derivatives of TIMP-3 with TIMP-3 biological activity may have amino acid sequences that consist of the amino acid sequence of the naturally occurring TIMP-3 protein or the amino acid sequence of naturally occurring TIMP-3 protein with

minor variations. TIMP-3 derivatives also include polypeptides with the amino acid sequence of TIMP-3 with one or more amino acid substitutions. Preferably, these amino acid substitutions are the result of the substitution of one amino acid with another amino acid having a similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine, i.e., conservative amino acid replacements. Furthermore, TIMP-3 derivatives may include polypeptides with the amino acid sequence of naturally occurring TIMP-3, but possessing various minor amino acid deletions and/or insertions, typically in the range of about 1 to 5 amino acids, as well as one or more amino acid substitutions. Guidance in determining which TIMP-3 amino acid residues may be replaced or deleted without abolishing TIMP-3 biological activities of interest may be found by searching computer libraries of protein amino acid sequences for proteins that are homologous to a given tissue inhibitor of metalloproteinases and minimizing the number of amino acid sequence changes in regions of homology between TIMP-3 and homologous proteins, such as TIMP-1 or TIMP-2 detected by a computer homology search. Additionally, TIMP-3 amino acid residues that may be replaced or deleted without abolishing TIMP-3 biological activities of interest may be determined by systematically making insertions, deletions, or replacement of TIMP-3 amino acids (by recombinant DNA techniques) and assaying for the biological activities of interest.

The term TIMP-3 refers to TIMP-3 that may or may not be post-translationally modified. Post-translational modification includes processes such as proteolytic cleavage (including the action of signal peptidases), glycosylation, lipidation, and the like. The nature of post-translational modification may vary in accordance with the type of cell in which TIMP-3 is produced.

Although the TIMP-3 genes is transcribed by endotoxin activated THP-1 cells, the naturally occurring expression of

TIMP-3 is not known to be limited to THP-1 cells or macrophages or monocytes.

A "mutation" in a protein alters its primary structure (relative to the commonly occurring or specifically described protein) due to changes in the nucleotide sequence of the DNA which encodes the protein. These mutations include allelic variants. Mutational changes in the primary structure of a protein result from deletions, additions or substitutions. A "deletion" is defined as a change in a polypeptide sequence in which one or more internal amino acid residues are absent. An "addition" is defined as a change in a polypeptide sequence which has resulted in one or more additional internal amino acid residues as compared to the wild type. A "substitution" results from the replacement of one or more amino acid residues by other residues. A protein "fragment" is a polypeptide consisting of a primary amino acid sequence which is identical to a portion of the primary sequence of the protein to which the polypeptide is related.

A host cell "expresses" a gene or DNA when the gene or DNA is transcribed and the protein encoded by the transcribed RNA is produced by translation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for nucleotide sequences uniquely identifying TIMP-3, nucleotide sequences encoding TIMP-3, and purified TIMP-3.

Nucleotide sequences encoding TIMP-3 have numerous applications in techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use for chromosome and gene mapping, use in the recombinant production of TIMP-3, generation of anti-sense RNA, DNA or nucleotide analogues, and the like. The uses of oligonucleotides encoding by TIMP-3 provided for in this application are exemplary of known techniques and are not intended to reflect any limitation on their use in any technique that would be known to the person of average skill in the art. Furthermore, the nucleotide sequences provided

for in this application may be used in molecular biology techniques that have not yet been developed, provided the new techniques rely on properties of polynucleotide sequences that are currently known to the person of average skill in the art, e.g., the triplet genetic code, specific base pair interactions, etc.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of TIMP-3-encoding nucleotide sequences, some bearing minimal nucleotide sequence homology to the nucleotide sequence of the naturally occurring TIMP-3 gene, may be produced. The invention has specifically contemplated each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring TIMP-3 encoding nucleotide sequences and all such variations are to be considered as being specifically disclosed.

Although nucleotide sequences of interest encoding TIMP-3 and TIMP-3 derivatives are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring TIMP-3 gene under stringent conditions, it may be advantageous to produce nucleotide sequences encoding TIMP-3 or TIMP-3 derivatives possessing a substantially different codon usage. Codons may be altered by chemical synthesis of the entire gene or relevant portions thereof, or by recombinant DNA methods including but not limited to site-directed mutagenesis (see generally, Ausubel, F.M. et al., in Current Protocols in Molecular Biology, John Wiley & Sons, all Vols., and periodic updates thereof, 1989). Codons can be selected to increase the rate at which expression of the protein or peptide occurs in a particular expression host organism in accordance with the frequency with which a particular codon is utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding TIMP-3 and/or a TIMP-3 derivative without altering the amino acid sequence include the production of RNA

transcripts having more desirable properties, .g., a greater half-life, than transcripts produced from the naturally occurring TIMP-3 encoding nucleotide sequence and the like.

Nucleotide sequences encoding TIMP-3 may be joined to a variety of other nucleotide sequences of interest by means of well established recombinant DNA techniques (see, for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor (1989).

Interesting nucleotide sequences for joining to TIMP-3 sequences include an assortment of cloning vectors, e.g., plasmids, cosmids, phage derivatives, phagemids, and the like, that are in the public domain. Vectors of interest include expression vectors, replication vectors, probe generation vectors, sequencing vectors, and the like. In general, vectors of interest may contain an origin of replication functional in at least one organism, convenient restriction endonuclease digestion sites, and selectable markers for the host cell.

Another aspect of the subject invention is to provide for TIMP-3-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences encoding TIMP-3. Nucleic acid hybridization probes for the detection of TIMP-3 encoding nucleotide sequences should preferably contain at least 50% of the nucleotides from the sequence of a given TIMP-3 encoding nucleotide sequence. The hybridization probes of the subject invention may be derived from the nucleotide sequences of the Sequence Listing and the genomic sequences encoding TIMP-3 uniquely identified by the nucleotide sequences of the Sequence Listing. Hybridization probes may be labeled by a variety of reporter groups, including radionuclides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase, coupled to the probe via avidin/biotin coupling systems, and the like.

An additional use for nucleic acid hybridization probes involves their use as primers for PCR, the polymerase chain reaction. The polymerase chain reaction is described in detail in U.S. Patents 4,965,188 and 4,683,195 and 4,800,195.

Probes for hybridization may be synthesized by both enzymatic and in vitro techniques. Short hybridization probes are preferably synthesized by in vitro methodology such as the use of commercially available DNA synthesizers like machines sold by Applied Biosystems. For example, nucleotide sequences of lengths greater than 10 base pairs may be produced by commercially available machines. Oligonucleotides produced by in vitro synthesis may be readily spliced together using generally known recombinant DNA techniques to produce a longer sequence of interest.

Other means of producing TIMP-3-specific hybridization probes include the cloning of nucleic acid sequences encoding TIMP-3 and TIMP-3 derivatives into vectors for the production of RNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase, or the like, and the appropriate radioactively labeled nucleotides.

It is now possible to produce a DNA sequence, or portions thereof, encoding TIMP-3 and TIMP-3 derivatives entirely by synthetic chemistry, after which the gene can be inserted into any of the many available DNA vectors using reagents, vectors and cells that are freely available and in the public domain at the time of the filing of this application. Synthetic chemistry may be used to reproduce the entire sequence of a TIMP-3 encoding gene, any portion thereof, or to introduce in mutations into the sequence.

The nucleotide sequence for TIMP-3 can be used to construct hybridization probes for mapping the TIMP-3 gene and for the genetic analysis of individuals with TIMP-3 genetic disorders, allelic variants or other genetic traits of interest. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosomes using well-known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries or flow-sorted

chromos mal preparati ns specific t known chromosomes, and the like. The technique of florescence in situ hybridization of chromosome spreads has been described, among other places, in Verma et al. Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988). Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques, may be correlated with additional genetic map data. Examples of genetic map data can be found, for example, in Genetic Maps: Locus Maps of Complex Genomes, Book 5: Human Maps, O'Brien, editor, Cold Spring Harbor Laboratory Press (1990). Correlation between a physical chromosomal map may be of particular interest in detecting genetic diseases in victims and potential carriers of genetic diseases. The nucleotide sequence of the subject invention may be used to detect differences in the sequence of genes that vary between normal individuals and affected individuals.

Nucleotide sequences encoding TIMP-3 may be used to produce purified TIMP-3 using well-known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Gene Expression Technology, Methods and Enzymology, Vol. 185, edited by Goeddel, Academic Press, San Diego, California (1990) TIMP-3 may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from species either the same or different than the species from which the TIMP-3 encoding nucleotide sequences are naturally present, i.e., endogenous. Advantages of producing the TIMP-3 by recombinant DNA technology include obtaining highly enriched sources of the proteins for purification and the availability of simplified purification procedures.

Cells transformed with expression vectors encoding TIMP-3 may be cultured under conditions favoring expression of the TIMP-3 sequence and the recovery of the recombinantly-produced protein from the cell culture. TIMP-3 produced by a recombinant cell may be secreted or may be contained

intracellularly, depending on the particular genetic construction used. In general, it is more convenient to prepare recombinant proteins in secreted form. Purification steps will depend on the nature of the production and the particular TIMP-3 produced.

In addition to recombinant production, TIMP-3 fragments may be produced by direct peptide synthesis using solid-phase techniques. See Stewart, et al., Solid-Phase Peptide Synthesis (1969), W. H. Freeman Co., San Francisco, and Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963).

In vitro polypeptide synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Foster City, California) following the instructions provided in the instruction manual supplied by the manufacturer.

Antibodies specific for a TIMP-3 may be produced by using TIMP-3 for the induction of said TIMP-3-specific antibodies. By induction of antibodies it is intended not only the stimulation of an immune response by injection into animals, but analogous steps in the production of synthetic antibodies or other specific-binding molecules such as the screening of recombinant immunoglobulin libraries, Orlandi et al., PNAS USA 86:3833-3837 (1989) or Huse et al., Science 256:1275-1281 (1989), or the in vitro stimulation of lymphocyte populations.

Current technology, e.g., Winter and Milstein, Nature, 349:293-299 (1991), provides for a number of highly specific binding reagents based on the principles of antibody formation; these techniques may readily be adapted to produce molecules capable of specifically binding TIMP-3.

TIMP-3 for use in the induction of antibodies of interest do not need to have biological activity; however, TIMP-3 for use in the induction of antibodies will necessarily have immunological activity. Polypeptides for use in the induction of TIMP-3-specific antibodies may have an amino acid sequence consisting of at least five amino acids, preferably at least 10 amino acids, mimicking a portion of the amino acid sequence

of TIMP-3, and may contain th ntire amino acid s quence of naturally occurring TIMP-3 or a TIMP-3 derivative.

An additional embodiment of the subject invention is the use of a composition comprising human TIMP-3, or derivatives thereof, as bioactive agents for the treatment of diseases including but not limited to: inflammation, arthritis, connective tissue disorders, periodontal disease, dermatological disorders, infection, ophthalmic disorders, corneal ulceration, orthopedic disorders, osteoporosis, autoimmune disease, and cancer. Compositions comprising TIMP-3 or derivatives thereof may also find application as ingredients or medicinal components of cosmetic or topical compositions.

When used in the therapeutic treatment of disease, an appropriate dosage of TIMP-3 or a TIMP-3 derivative may be determined by any of several well established methodologies. For instance, animal studies are commonly used to determine the maximal tolerable dose, or MTD, of bioactive agent per kilogram weight of the test subject. In general, at least one animal species tested is mammalian. Those skilled in the art regularly extrapolate doses for efficacy and avoiding toxicity to other species, including human. Before human studies of efficacy are undertaken, Phase I clinical studies in normal subjects help establish safe doses.

Additionally, the bioactive agent may be complexed with a variety of well established compounds or structures that, for instance, enhance the stability of the bioactive agent, or otherwise enhance its pharmacological properties (e.g., increase in vivo half-life, reduce toxicity, etc.).

Where diagnostic, therapeutic or medicinal use of TIMP-3 or derivatives thereof is contemplated, the bioactive agent may be introduced in vivo by any of a number of established methods. For instance, the agent may be administered by inhalation; by subcutaneous (sub-q); intravenous (I.V.), intraperitoneal (I.P.), intramuscular (I.M.) injection; or as a topically applied agent (transderm, ointments, creams, salves, eye drops, and the like).

The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention.

EXAMPLES

I. CONSTRUCTION OF cDNA LIBRARIES

The human monocyte THP-1 cDNA library was custom constructed by Stratagene (Stratagene, 11099 M. Torrey Pines Rd., La Jolla, CA 92037). Poly(A+)RNA (mRNA) was purified from THP-1 cells (cultured 48 hr with 100nm TPA and 4 hr with 1μg/ml LPS). cDNA synthesis was primed separately with both oligo dT and random hexamers and the two cDNA libraries were treated separately. Synthetic adaptor oligonucleotides were ligated onto cDNA ends enabling its insertion into Uni-ZAP™ vector system (Stratagene), allowing high efficiency unidirectional (sense orientation) lambda library construction and the convenience of a plasmid system with blue/white color selection to detect clones with cDNA insertions. Finally, the two libraries were combined into a single library by mixing equal numbers of bacteriophage.

The THP-1 cDNA library can be screened with either DNA probes or antibody probes and the pBluescript® phagemid (Stratagene) can be rapidly excised *in vivo*. The phagemid allows the use of a plasmid system for: easy insert characterization, sequencing, site-directed mutagenesis, the creation of unidirectional deletions and expression of fusion proteins. The custom-constructed library phage particles-were infected into *E. coli* host strain XL1-Blue® (Stratagene), which has a high transformation efficiency, increasing the probability of obtaining rare, under-represented clones in the cDNA library.

II. ISOLATION OF cDNA CLONES

The phagemid forms of individual cDNA clones were obtained by the *in vivo* excision process, in which the host

bacterial strain was coinf cted with both the lambda library phage and an f1 h lper phage. Proteins derived from both the library-containing phage and the helper phage nicked the lambda DNA, initiated new DNA synthesis from defined sequences on the lambda target DNA and created a smaller, single stranded circular phagemid DNA molecule that included all DNA sequences of the pBluescript® plasmid and the cDNA insert. The resulting phage containing the phagmid DNA were secreted from the cells, purified, and then used to re-infect fresh host cells, where the double stranded phagemid DNA was produced. Because the phagemid carries the gene for β-lactamase, the newly-transformed bacteria are selected on medium containing ampicillin.

Phagemid DNA was purified using the Magic Minipreps™ DNA Purification System (Promega catalogue #A7100. Promega Corp., 2800 Woods Hollow Rd., Madison, WI 53711). This small-scale process provides a simple and reliable method for lysing the bacterial cells and rapidly isolating purified phagemid DNA using a proprietary DNA-binding resin. The DNA was eluted from the purification resin already prepared for DNA sequencing and other analytical manipulations.

Phagemid DNA was also purified using the QIAwell-8 Plasmid Purification System from QIAGEN® DNA Purification System (QIAGEN Inc., 9259 Eton Ave., Chattsworth, CA 91311). This product line provides a convenient, rapid and reliable high-throughput method for lysing the bacterial cells and isolating highly purified phagemid DNA using QIAGEN anion-exchange resin particles with EMPORE™ membrane technology from 3M in a multiwell format. The DNA was eluted from the purification resin already prepared for DNA sequencing and other analytical manipulations.

III. SEQUENCING OF cDNA CLONES

The cDNA inserts from random isolates of the THP-1 library was sequenced in part. Methods for DNA sequencing are well known in the art. Conventional enzymatic methods employ DNA polymerase Klenow fragment, Sequenase™ or Taq polymerase

to extend DNA chains from an oligonucleotide primer annealed to the DNA template of interest. Methods have b en developed for the use of both single- and double stranded templates. The chain termination reaction products are usually electrophoresed on urea-polyacrylamide gels and are detected either by autoradiography (for radionuclide-labelled precursors) or by fluorescence (for fluorescent-labelled precursors). Recent improvements in mechanized reaction preparation, sequencing and analysis using the fluorescent detection method have permitted expansion in the number of sequences that can be determined per day (such as the Applied Biosystems 373 DNA sequencer and Catalyst 800).

IV. HOMOLOGY SEARCHING OF cDNA CLONE AND DEDUCED PROTEIN

Using the nucleotide sequences derived from the cDNA clones as query sequences (the sequences of the Sequence Listing), databases containing previously identified sequences are searched for areas of homology (similarity). Such databases include Genbank and EMBL. Two homology search algorithms were used.

The first algorithm was originally developed by D.J. Lipman and W.R. Pearson, "Rapid and Sensitive Protein Similarity Searches", Science, 227, 1435 (1985). In this algorithm, the homologous regions are searched in a two step manner. In the first step, the highest homologous regions are determined by calculating a matching score using a homology score table. The parameter 'Ktup' is used in this step to establish the minimum window size to be shifted for comparing two sequences. Ktup also sets the number of bases that must match to extract the highest homologous region among the sequences. In this step, no insertions or deletions are applied and the homology is displayed as an initial (INIT) value.

In the second step, the homologous regions are aligned to obtain the highest matching score by inserting a gap in order to add a probable deleted portion. The matching score obtained in the first step is recalculated using the homology

sc re Table and the insertion scor Table to an optimiz d (OPT) value in the final output.

DNA homologies between two sequences can be examined graphically using the Harr method of constructing dot matrix homology plots (Needleman, S.B. and Wunsch, C.O., J. Mol. Biol 48:443 (1970)). This method produces a two-dimensional plot which can be useful in determining regions of homology versus regions of repetition.

The second algorithm was developed by Applied Biosystems Inc. and has been incorporated into the Inherit 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (developed by TRW Inc.) is used to determine regions of homology. There are three parameters that determine how the sequence comparisons are run: window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database is searched for sequences containing regions of homology and the appropriate sequences are scored with an initial value. Subsequently, these homologous regions are examined using dot matrix homology plots to determine regions of homology versus regions of repetition. Smith-Waterman alignments were used to display the results of the homology search.

Following the search for homologous regions, the sequences from the cDNA clones were classified as to whether they are exact matches (regions of exact homology) homologous human matches (regions of high similarity, but not exact matches), homologous non-human matches (regions of high similarity present in species other than human), or nonmatches (no significant regions of homology to previously identified nucleotide sequences).

Searches of the deduced proteins and peptides are done in a manner analogous to that done with the cDNA sequences. The sequence of the protein is used as a query sequence and compared to the previously identified sequences contained in a database such as Swiss/Prot or the NBRF Protein database to find homologous proteins. These proteins are initially scored for homology using a homology score Table (Orcutt, B.C. and

Dayhoff, M.O. Scoring Matrices, PIR Report MAT - 0285 (February 1985)) resulting in an INIT score. The homologous regions are aligned to obtain the highest matching scores by inserting a gap which adds a probable deleted portion. The matching score is recalculated using the homology score Table and the insertion score Table resulting in an optimized (OPT) score. Even in the absence of knowledge of the proper reading frame of an isolated sequence, the above-described protein homology search may be performed by searching all 3 reading frames.

Peptide and protein sequence homologies can also be ascertained using the Inherit 670 Sequence Analysis System in an analogous way to that used in DNA sequence homologies. Pattern Specification Language and parameter windows are used to search protein databases for sequences containing regions of homology which are scored with an initial value. Subsequent examination with a dot-matrix homology plot determines regions of homology versus regions of repetition.

V. IDENTIFICATION OF TIMP-3 CDNA

Using the methodology described above, Incyte template #12006 was found to be homologous to members of the TIMP family, a family of homologous proteins which are known to be inhibitors of the matrix metalloproteinases. When raw sequence data was analyzed for homology with the GENBANK database, the best match was found to be with GENBANK entry CHKHIMP3A, the entry for a chicken TIMP. Template #12006 was also found to be homologous to the remaining several members of the TIMP family from a variety of species. The Document Hit scores for all homologies detected were low, identifying template #12006 as a homolog for the database entries rather than an exact match. The homology between human TIMP-3 and human TIMP-1 and TIMP-2 as well as chicken TIMP-3 is shown in Table 1. Other templates have been exact matches to previously know human TIMPs. Subsequently, two other template sequences (#12417 and #12777) were identified which also showed homology to the TIMP family of protease inhibitors.

VI. ANTISENSE ANALYSIS

Knowledge of th correct, complete cDNA sequence of the novel TIMP-3 gene will enable its use in antisense technology in the investigation of its gene function. Either oligonucleotides, gene or cDNA fragments encoding the antisense strand of the TIMP-3 gene can be used either *in vitro* or *in vivo* to inhibit a specific gene function. By treatment of cells or whole test animals with such antisense probes, the gene of interest can be effectively turned off. Frequently, the function of the genes of interest can be ascertained by observing cellular or organismic behavior (e.g. lethality, loss of differentiated function, changes in mobility or morphology, changes in surface antigens).

By the same methodology, the expression of a gene whose function it may be desirable to inhibit or turn-off may be manipulated. For example, a new gene whose function is related to a human disorder can be treated therapeutically by administration (either orally or intravenously) of antisense DNA, RNA or nucleotide derivatives which hybridize to DNA to shut off transcription of the offending or diseased gene. Such technology is now well known in the art, and can be designed at various locations within the coded gene sequence. Similarly, such inhibition cans be achieved using Hogeboom base-pairing methodology, also known as "triple helix" base pairing. In addition to the gene itself, modifications of gene expression can be obtained by designing antisense probes to extragenic regions such as promoter regions, enhancer regions, or even to other genes which control or inhibit the synthesis of the gene of interest.

VII. EXPRESSION OF cDNA CLONES

Translation of cloned TIMP-3 cDNA into protein may be accomplished by subcloning the cDNA into an appropriate expression vehicle and subsequently into an appropriate expression host. In this particular case, the library cloning vector used for the generation of the THP-1 library includes provisions for direct expression of the included sequence in

E. coli: upstream of the cloning site lies a promoter for β-galactosidase, followed by the amino-terminal Met and subsequent 7 residues of β-galactosidase. Immediately following this is an engineered bacteriophage promoter (useful for artificial priming and transcription), and other included unique restriction sites, up to and including the Eco RI cloning site and subsequent cDNA insert. Since cDNA clone inserts are generated by an essentially random process, there is a one-in-three chance that the included cDNA will lie in the correct frame for proper translation, and that induction of the isolated bacterial strain with IPTG using standard methods will produce a fusion protein corresponding to the first seven residues of B-galactosidase, some 15 residues of "linker", and the peptide encoded within the cDNA. There is also a two in three chance the cDNA will lie in the wrong reading frame. If the cDNA is not in the proper reading frame, the proper reading frame can be obtained by deletion or insertion of the appropriate bases by well known methods including in vitro mutagenesis, nuclease digestion, or oligonucleotide linker inclusion, and used subsequently for bacterial expression studies as described.

Oligonucleotide amplimers containing cloning sites as well as a segment of DNA sufficient to hybridize to stretches at both ends of the target cDNA (25 bases) can be synthesized chemically by standard methods. These primers can then used to amplify the desired gene segments by Polymerase Chain Reaction (PCR). The resulting new gene segments can be digested with the corresponding restriction enzyme under standard conditions and isolated by gel electrophoresis. Alternately, similar gene segments can be produced by digestion of the cDNA itself with appropriate restriction enzymes and filling in missing gene segments with chemically synthesized oligonucleotides. These segments of coding sequence can be ligated together and cloned into appropriate expression vectors which would allow recombinant production of the encoded sequence. Suitable expression systems for such chimeric molecules include but are not limited to mammalian

c lls such as CHO c lls, fungi, such as yeast, insect cells and viruses, such as Baculovirus, and bacteria such as E. coli. For each of these cell systems, a useful expression vector may include a selectable phenotypic marker, e.g., an antibiotic resistance gene, such as ß-lactamase, that would allow selection of recombinant clones; a second selectable marker to allow selection on the host cells containing the expression vector, such as neomycin phosphotransferase; replication origin to allow propagation in bacteria; a high level promoter driving a gene inserted into an insertion site, such as retroviral long terminal repeat (LTR) promoter/enhancers, the MMTV, SV40, or metallothionine promoter for CHO cells, the trp, lac, tac or T7 promoter for bacterial hosts, or the alpha factor or PGH promoters in yeast; transcription enhancers for the mammalian hosts, such as the RSV enhancer; and a polyadenylation site, AATAAA, if one does not exist within the cDNA sequence. Once homogeneous cultures of recombinant cells are obtained through standard culture methods, large quantities of recombinantly produced TIMP-3 can be recovered and analyzed from the conditioned medium through standard chromatographic methods.

VIII. ISOLATION OF RECOMBINANTLY PRODUCED TIMP-3

TIMP-3 may be expressed recombinantly in such a way as to facilitate purification of the TIMP-3 protein. Such an approach involves expression of the TIMP-3 as a chimeric protein with one or more additional polypeptide modules; these modules provide for a general method for purification of the chimeric proteins. The term "module" refers to a domain of a protein not naturally present on naturally occurring TIMP-3. Such purification facilitating modules include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification utilizing immobilized metals, protein A extensions that allow purification on immobilized immunoglobulin, and the FLAGS extension/affinity purification system (Immunex Corp., Seattle WA). The inclusion of a linker sequence (such as Factor XA or

enterokinase) upstream of the TIMP-3-encoding sequence may be us ful to facilitate subsequent cleavage of the intact TIMP-3-protein.

IX. PRODUCTION OF ANTI-TIMP-3 ANTIBODIES

Two approaches are utilized to raise antibodies to TIMP-3 and both approaches can be used to generate either polyclonal or monoclonal antibodies. In one approach, a denatured protein from the reverse phase HPLC separation is obtained in quantities up to 75 mg. This denatured protein can be used to immunize mice or rabbits using standard protocols; about 100 micrograms are adequate for immunization of a mouse, while up to 1 mg might be used to immunize rabbit. For screening mouse hybridomas, the denatured protein can be radioiodinated and used to screen murine B-cell hybridomas for those which produce antibody. This procedure requires only small quantities of protein, such that 20 mg would be sufficient for labeling and screening of several thousand clones.

In the second approach, the amino acid sequence of TIMP-3, as deduced from the TIMP-3-cDNA, is analyzed to determine regions of high immunogenicity. The corresponding polypeptides are synthesized and are used in suitable immunization protocols to raise antibodies. Analysis to select appropriate epitopes is described by, for example, Ausubel, F.M. et al., in Current Protocols in Molecular Biology, John Wiley & Sons, Vol. 2, Sec. IV, pp 11.14.1, 1989. The optimal amino acid sequences for immunization are usually at the C-terminus, the N-terminus and internal regions of the polypeptide, which are likely to be exposed to the external nvironment when the protein is in its natural conformation (usually the most hydrophobic regions of the molecule). Typically, selected peptides, about 15 residues in length, are synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry and coupled to keyhole limpet hemocyanin (KLH, Sigma) by reaction with M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) (See Ausubel et al, supra at pp 11.15.1). If necessary, a cysteine

may be introduc d at the N-t rminus of the peptid t permit coupling to KLH. Rabbits are immunized with the peptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with antisera, washing and reacting with radioiodinated affinity purified specific goat anti-rabbit IgG.

Hybridomas may be also be prepared and screened using standard techniques. Hybridomas of interest are detected by screening with radioiodinated TIMP-3 to identify those fusions producing the monoclonal antibody with the desired specificity. In a typical protocol, wells of plates (FAST, Becton-Dickinson, Palo Alto, CA), are coated with affinity purified specific rabbit-anti-mouse (or suitable anti-species Ig) antibodies at 10 mg/ml. The coated wells are blocked with 1% BSA, washed and exposed to supernatants from hybridomas. After incubation the wells are exposed to radiolabeled TIMP-3, 1 mg/ml. Clones producing antibodies will bind a quantity of radioactivity which is detectable above background. Such clones are expanded and subjected to 2 cycles of cloning at 0.3 cells/well. Cloned hybridomas are injected into pristine treated mice to produce ascites, and monoclonal antibody is purified from the ascitic fluid by affinity chromatography on Protein A.

X. SCREENING OF ANTI-TIMP-3 ANTIBODIES AGAINST HUMAN MONOCYTE/MACROPHAGE CELLS

Some TIMP-3 specific antibodies may be useful for the diagnosis of prepathologic conditions, as well as the diagnosis of chronic and acute diseases that are characterized by abnormalities in the amount or distribution of TIMP-3. A variety of protocols for immunoassays, using either polyclonal or monoclonal antibodies specific for TIMP-3, are known in the art, e.g., competitive binding assays and immunoradiometric assays. Immunoassays typically involve the formation of complexes between TIMP-3 and a TIMP-3-specific antibody (or similar TIMP-3-specific molecule) and the measurement of complex formation. A two-site monoclonal-based immunoassay

utilizing monoclonal antibodies reactive to two noninterfering epitopes on TIMP-3 is preferred, but a comp titive binding assay may also be employed. These assays are described, among other places, in Maddox, D.E. et al, J. Exp. Med. (1983) 158:1211.

Immunoassay procedures are utilized to measure several major parameters in immunopathologic and prepathologic conditions which are characterized by TIMP-3 abnormalities, e.g., the increased or decreased production of TIMP-3 by monocytes/macrophages, the aberrant production of TIMP-3 by cells other than human monocytes/macrophages, and the change in intracellular or extracellular distribution of TIMP-3 during the genesis of disease. In order to determine the normal distribution of TIMP-3 in monocytes, peripheral blood mononuclear cells from normal individuals are prepared using Ficoll-Paque (Pharmacia) and analyzed as described by Marra et al. (J. Immun. 148: 532-537, 1971). The quantity of TIMP-3 in monocyte/macrophages may be determined by performing immunoassays on freeze-thawed detergent extracts of cell suspensions enriched for monocyte/macrophages and comparing the slope of the binding curves to comparable binding curves generated by the purified protein.

XI. PURIFICATION OF NATIVE TIMP-3 USING ANTI-TIMP-3 ANTIBODIES

Native or recombinant TIMP-3 may be purified by immunoaffinity chromatography using TIMP-3-specific antibodies. In general, an immunoaffinity column is constructed by covalently coupling the anti-TIMP-3 antibody to an activated chromatographic resin.

Polyclonal immunoglobulins are prepared from immune sera ither by precipitation with ammonium sulfate or by purification on immobilized Protein A (Pharmacia LKB Biotechnology, Piscataway, NJ). Likewise, monoclonal antibodies are prepared from mouse ascites fluid by ammonium sulfate precipitation or chromatography on immobilized Protein A. Partially purified Ig is covalently attached to a chromatographic resin, for example CnBr activated Sepharose

(Pharmacia LKB Biotechnology, Piscataway, NJ). Th antibody is coupled to the resin, the resin is blocked, and the derivative resin is washed according to the manufacturers instructions.

Such an immunoaffinty column is utilized in the purification of TIMP-3 by preparing a fraction from cells containing TIMP-3 in a soluble form. This preparation may be derived by solubilization of the whole cell, e.g., by the addition of detergent, or by the similar solubilization of a subcellular fraction by well known methods, e.g., differential centrifugation. Alternatively, the soluble TIMP-3 may be secreted into the medium conditioned by growth of cells that express TIMP-3 in useful quantity.

A soluble TIMP-3 containing preparation is passed over the immunoaffinity column and the column is washed under conditions that allow the preferential absorbance of TIMP-3, for example in high ionic strength buffers in the presence of detergent. The column is then eluted under conditions that disrupt antibody/TIMP-3 binding, e.g., a buffer of pH 2-3 or the presence of high concentration of a chaotrope e.g. urea or the thiocyanate ion.

XII. TIMP-3 FULL LENGTH CLONING AND CHARACTERIZATION

Three clones, 12006, 12417, and 12777 were discovered to contain TIMP-3 encoding sequence, and overlapped each other, implying discrete reverse transcriptase termination sites. The clone extending furthest in the 5'-direction, 12006, initiated 324 bases upstream from the stop codon, and therefore was incomplete at the 5' end. The cDNA insert from clone 12006 was excised from the clone DNA via restriction enzyme digestion, and was used as a hybridization probe against the THP-1 library. Hybridized under normal stringency conditions, 19 clones were detected by autoradiography. All 19 clones were selected for further analysis as follows: Each clone and its 10-20 neighboring clones (picking the correct individual at high density would be very difficult) were removed from the agar plate and soaked in 1 ml of SM buffer.

The DNA recovered was used as a target for PCR amplification with two specific amplimers: One amplimer corresponded to DNA sequence of the T3 promoter (5'-AGC TCG AAA TTA ACC CTC ACT AAAG-3') in the cloning vector immediately upstream from the insert itself. The other was complementary to bases 12 through 38 of the clone 12006 sequence (5'-TAC ATC CAT ACG GAA GTT TCC GAG AGT-3'). When the PCR products were analyzed by gel electrophoresis, the lengths of the sequences between the clone 12006 sequence and the start of the clone insert could be ascertained. The clone (IMP1) showing the longest PCR product (about 600 nucleotides) was purified to homogeneity, excised, amplified, extracted and sequenced by standard methods. The sequence included all of the putative amino acid coding regions for TIMP-3, and is shown as Sequence ID No 1 in the Sequence Listing.

XIII. NORTHERN AND SOUTHERN BLOTS

A probe was prepared by labeling insert from clone 12006 to a specific activity of approximately 1 x 10^9 cpm/μg, using α-^{32}P dCTP (3000Ci/mmol, Amersham, Arlington Heights, IL) with a commercially available random primer labeling kit (Promega Corp., 2800 Woods Hollow Rd., Madison, WI 53711). Multi-Tissue Northern (MTN) blots and the genomic Southern blot (GENO-BLOT™) were obtained from Clontech, Inc., Palo Alto, CA. These and the chromosomal localization blot (BIOSMAP™; BIOS Inc., New Haven, CT) were probed under standard high stringency conditions (hybridization: 5xSSPE, 10x Denhardt's, 0.1 mg/ml herring sperm DNA, 50% formamide, 2% SDS, 42°C; wash: 60°C, 0.2xSSC, 0.1% SDS). All northern and Southern exposures used Kodak XAR-2 film with 2 L-PLUS intensifying screens (Fisher Biotech Inc., Pittsburgh, PA) at -70°C.

Tissue-specific transcription of TIMP-3 was examined by RNA blot analysis using 3 ug poly A+ RNA per lane. A PCR-generated TIMP-3 clone insert was used as the hybridization probe. Figures 10 A and 10 B contain normal adult tissues; Fig. 10 C contains human fetal tissues. The lanes correspond to spleen (1), thymus (2), prostate (3), testis (4), ovary

(5), small int stine (6), colon (7), peripheral blood leukocyte (8), heart (9), brain (10), placenta (11), lung (12), liver (13), skeletal muscle (14), kidney (15), pancreas (16), fetal heart (17), fetal brain (18), fetal lung (19), fetal liver (20), and fetal kidney (21). Molecular weight standards are shown along the left side of the blot.

Transcripts of 5.0, 2.6, and 2.4 kB were detected in all tissues examined and the 5.0 kB band represented at least 90% of the hybridization signal in most tissues. The ratio between the three bands seemed relatively invariant, except for in the placental samples, where the relative levels of the 2.6 and 2.4 kB bands were much greater. The strongest hybridization signals were found in spleen, prostate, ovary, small intestine, heart, placenta, lung, and kidney. Significantly less TIMP-3 mRNA was detectable in colon, peripheral blood leukocytes, brain, liver, and pancreas although all three bands were clearly present. In general, the profile was similar for fetal tissues; however, both fetal heart and liver contained increased levels of TIMP-3 mRNA relative to adult tissue. When the same filters were stripped and reprobed with actin cDNA, similar mRNA levels were detected in all 21 tissue samples.

In Figure 11, TIMP-3 cDNA was used as a hybridization probe against blots of human chromosomal DNA digested with Eco RI (lane 2), Hind III (lane 3), Bam HI (lane 4), Pst I (lane 5), and Bgl II (lane 6). Molecular weight standards are shown along the left side of the blot. The signal pattern indicates that TIMP-3 mRNA originates from a very small number of genes, most likely a single gene. The DNA hybridization patterns present no evidence that TIMP-3 is a member of a multigene family or even closely related to other genes. In Figure 12, the TIMP-3 probe was hybridized to a blot of Eco RI-digested chromosomal DNA from 20 mouse/human and hamster/human somatic cell hybrids. Such blots are often useful for assigning genes to individual human chromosomes. Included are hamster DNA (lanes 1 and 15), human DNA (lane 2 and 16), mouse DNA (lane 13), molecular weight markers (lanes 14 and

28), and hybrids (lanes 3-11, 17-27). Lane 8 represents chromosomes 1, 5, 12, 14,19, 21, and 22; lane 17, chromosomes 4, 5, 8, 22, X; lane 27, chromosomes 1, 5, 13, 19, 21, and 22. The 2.4 kB hybridization signals that correspond to the human gene were detected in lanes 8, 17, and 27. This pattern is consistent only with localization on chromosome 5 or 22. Since no signal was detected in several other lanes containing the DNA of chromosome 5, TIMP-3 could be localized to chromosome 22.

In Figure 13 a "zoo" blot (ZOO-BLOT™; Clontech, Inc., Palo Alto, CA) was probed with TIMP-3 cDNA under medium stringency conditions (as above except hybridization: 37°C; wash: 1xSSC at 60°C). The "zoo" blot contains EcoRI-digested chromosomal DNA from various species; human (lane 2), rhesus monkey (lane 3), rat (lane 4), mouse (lane 5), dog (lane 6), cow (lane 7), rabbit (lane 8), chicken (lane 9) and Saccharomyces cerevisiae; and it is used to determine the degree of conservation of the primary nucleotide sequence. Lane 1 contained molecular weight standards.

Signals of similar intensity and size were seen in lanes corresponding to human, monkey, dog, and cow. A distinct band f smaller size was visible in mouse DNA (lane 5). No discrete bands could be discerned in the lanes corresponding to rat, rabbit, chicken or yeast DNA.

XIV. CONSTRUCTION OF THE EXPRESSION VECTOR

The full length cDNA for TIMP-3 including the native leader sequence was cloned into pCEP-4 (Invitrogen, San Diego, CA) using standard PCR techniques. pCEP-4 was introduced into competent E. coli strain DH5-α using standard heat-shock procedures. Amplified plasmid DNA was isolated, purified using the Qiagen® DNA Purification System (Qiagen, Chattsworth, CA 91311), and used to transfect appropriate host cells such as Chinese Hamster ovary (CHO), human 293 cells, Sf9 insect cells, or yeast, Saccharomyces cerevisiae, cells.

XV. EXPRESSION OF TIMP-3 IN 293-EBNA HOST CELLS

H st cells, 293-EBNA (Invitrog n, San Diego, CA) were transformed with the expression plasmid containing the TIMP-3 gene by lipofection (Gibco BRL, Grand Island, NY) according to standard protocol. Cells harboring plasmids were selected in REM (JRH Biosciences, Woodland, CA) or other similar basal medium such as DMEM/F12 (Gibco BRL), 10% calf serum, and 2 mM L-glutamine supplemented with 250 μg/ml geneticin (Gibco BRL) and 400 μg/ml hygromycin B (Calbiochem, La Jolla, CA). Transformed cell numbers were increased in the same selective medium in T-flasks (Costar, Cambridge, MA) and 500 ml spinner flasks (Corning, Corning, NY). The protein, TIMP-3, was secreted from cells grown in modified 3L Bellco spinner flasks (Bellco, Vineland, NJ). Cells were harvested from the conditioned medium by centrifugation.

XVI. TIMP-3 ZYMOGRAPHY

Because chicken TIMP-3 is known to bind to extracellular matrix (ECM), human TIMP-3 was tested for its ability to do the same. ECM was extracted from the surface of culture vessels upon which THP-1 cells had grown and also from the outer membrane of THP-1 suspension culture cells. Aliquots of conditioned media, previously discussed above, and ECM from both vessels and suspension culture cells were electrophoresed to separate their various proteins.

The resulting gel was soaked in collagen and subsequently digested with collagenase. The bands which were evident on the resulting zymogram are those of peptides that inhibit c llagenase. TIMP-3 presence, size, and activity were verified using this reverse zymography process (Tyagi, S.C. et al (1993) Mol. Cell. Biol. 126:49-59).

XVII. PRODUCTION OF ANTIBODIES TO TIMP-3 PROTEIN

Antibodies were produced against peptides form two different hydrophilic regions of TIMP-3. These peptides were synthesized on an Applied Biosystems Peptide Synthesizer model 431A using Fmoc chemistry as specified by the manufacturer.

The first synthetic peptide covered TIMP-3 amino acids 20 to 33, or RAKVVGKKLVKEG. The second synthetic peptide covered TIMP-3 amino acids 172-187, or YRGWAPPDKSIINATD.

The synthetic peptides, TIMP-3/20-33 and TIMP-3/172-187, were conjugated to the carrier protein, keyhole limpet hemocyanin (KLH) using the Pierce Imject® Activated Immunogen Conjugation Kit. The KLH-conjugates were injected into female New Zealand white rabbits. Two booster inoculations were given at two and four weeks after the primary injection. Antisera were collected and titered against BSA-TIMP-3 conjugates using standard ELISA protocol. Both TIMP-3 antisera preparations had titers of 1:8000.

All publications and patents mentioned in the above specification are herein incorporated by reference. The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Indeed, various modifications of the above-described modes for carrying out the invention which are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.

TABLE 1

Amino Acid Homology Analysis of TIMP-1,2,3 from Humans

Chicken	TIMP-3					-	-	M	T	A	W	L	G	F	L	A	V	F	L	C	S	W	S	L	R	D	L	V	A	E	A
Human	TIMP-1					-	-	-	M	A	P	F	E	P	L	A	S	G	I	L	L	L	L	W	L	I	A	P	S	R	A
Human	TIMP-2					M	G	A	A	A	R	T	L	R	L	A	L	G	I	L	L	L	A	T	L	L	R	P	A	D	A
Human	TIMP-3					-	-	M	T	P	W	L	G	-	L	I	V	L	L	G	S	W	S	L	G	D	W	G	A	E	A
Chicken	TIMP-3	C	T	C	V	P	I	H	P	Q	D	A	F	C	N	S	D	I	V	I	R	A	K	V	V	G	K	K	L	M	K
Human	TIMP-1	C	T	C	V	P	P	H	P	Q	T	A	F	C	N	S	D	L	V	I	R	A	K	F	V	G	T	P	E	V	N
Human	TIMP-2	C	S	C	S	P	V	H	P	Q	Q	A	F	C	N	A	D	V	V	I	R	A	K	A	V	S	E	K	E	V	D
Human	TIMP-3	C	T	C	S	P	S	H	P	Q	D	A	F	C	N	S	D	I	V	I	R	A	K	V	V	G	K	K	L	V	K
Chicken	TIMP-3	D	G	-	-	-	-	-	-	P	F	G	T	M	R	Y	T	V	K	Q	M	K	M	Y	R	G	F	Q	I	-	-
Human	TIMP-1	Q	-	-	-	-	-	-	-	T	T	L	Y	Q	R	Y	E	I	K	M	T	K	M	Y	K	G	F	Q	A	L	G
Human	TIMP-2	S	G	N	D	I	Y	G	N	P	I	K	R	I	Q	Y	E	I	K	Q	I	K	M	F	K	G	P	E	-	-	-
Human	TIMP-3	E	G	-	-	-	-	-	-	P	F	G	T	L	V	Y	T	I	K	Q	M	K	M	Y	R	G	F	T	K	-	-
Chicken	TIMP-3	-	M	P	H	V	Q	Y	I	Y	T	E	A	S	B	S	L	C	G	-	V	K	L	E	V	-	N	K	Y	Q	Y
Human	TIMP-1	D	A	A	D	I	R	F	V	Y	T	P	A	M	E	S	V	C	G	Y	F	H	R	S	H	N	R	S	E	E	F
Human	TIMP-2	-	-	K	D	I	E	F	I	Y	T	A	P	S	S	A	V	C	G	-	V	E	L	D	V	G	G	K	K	E	Y
Human	TIMP-3	-	M	P	H	V	Q	Y	I	H	T	E	A	S	E	S	L	C	G	-	L	K	L	E	V	-	N	K	Y	Q	Y
Chicken	TIMP-3	L	I	T	G	R	V	Y	E	G	K	V	Y	T	G	L	C	N	W	Y	E	K	W	D	R	L	T	L	S	Q	R
Human	TIMP-1	L	I	A	G	K	L	Q	D	G	L	L	H	I	T	T	C	S	F	V	A	P	W	N	S	L	S	L	A	Q	R
Human	TIMP-2	L	I	A	G	K	A	E	D	G	K	A	H	I	T	L	C	D	F	I	V	P	W	D	T	L	S	T	T	Q	K
Human	TIMP-3	L	L	T	G	R	V	Y	D	G	K	M	Y	T	G	L	C	N	F	V	E	R	W	D	Q	L	T	L	S	Q	R
Chicken	TIMP-3	K	G	L	N	H	R	Y	H	L	G	C	-	G	C	K	I	R	P	C	Y	Y	L	P	C	F	A	T	S	K	N
Human	TIMP-1	R	G	F	T	K	T	Y	T	V	G	C	E	E	C	T	V	F	P	C	L	S	I	P	C	K	L	Q	S	G	T
Human	TIMP-2	K	S	L	N	H	R	Y	Q	Q	G	C	-	E	C	K	I	T	R	C	P	M	I	P	C	Y	I	S	S	P	D
Human	TIMP-3	K	G	L	N	Y	R	Y	H	L	G	C	-	N	C	K	I	K	S	C	Y	Y	L	P	C	F	V	T	S	K	N
Chicken	TIMP-3	E	C	I	W	T	D	M	L	S	N	F	G	H	S	G	H	Q	A	K	H	Y	A	C	I	Q	R	V	E	G	Y
Human	TIMP-1	H	C	L	W	T	D	Q	L	L	Q	G	S	E	K	G	F	Q	S	R	H	L	A	C	L	P	R	E	P	G	L
Human	TIMP-2	E	C	L	W	T	D	W	V	T	E	K	M	I	N	G	H	Q	A	K	F	F	A	C	I	K	A	S	D	G	S
Human	TIMP-3	E	C	L	W	T	D	M	L	S	N	F	G	Y	P	G	Y	Q	S	K	H	Y	A	C	I	R	Q	K	G	G	Y
Chicken	TIMP-3	C	S	W	Y	R	G	W	A	P	P	D	K	T	I	I	N	A	T	D	P											
Human	TIMP-1	C	T	W	Q	S	L	R	S	Q	I	A																				
Human	TIMP-2	C	A	W	R	R	G	A	A	P	P	K	Q	E	F	L	D	I	E	D	P											
Human	TIMP-3	D	S	W	Y	R	G	W	A	P	P	D	K	S	I	I	N	A	T	D	P											

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Incyte Pharmaceuticals, Inc.
3330 Hillview Avenue
Palo Alto, California 94304
United States of America

(ii) TITLE OF INVENTION: Novel Human Monocyte/Macrophage-Derived Metalloproteinase, Its Production and Uses

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: USA
(F) ZIP: 10036-2711

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy Disk
(B) COMPUTER: IBM PC Compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: MS-DOS/Wordperfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT To be assigned
(B) FILING DATE: 07-OCT-1994

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/133,956
(B) FILING DATE: 07-OCT-1993

ATTORNEY/AGENT INFORMATION:
(A) NAME: Albert P. Halluin
(B) REGISTRATION NUMBER: 25,227
(C) REFERENCE/DOCKET NUMBER: 8135-009-228

(viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415-854-3660
(B) TELEFAX: 415-854-3694
(C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: T-012006

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CCGGGAGGCC GCCCGCCGAG TCCTGCGCCA GGCCGAGGCA GCCTCGCTGG CCCCATCCCG   120

TCCCGCCGGG CACTCGGAGG GCAGCGCGCC GGAGGNCAAG GTTGCCCCGC ACGGCCCGGC   180

GGGCGAGCGA GCTCGGGCTG CAGCAGCCCC GCCGGNNNNC GCACGGCAAC TTTGGAGAGG   240

CGAGCAGCAGC CCCGGCAGC GGCGGCAGCA GCGGCA ATG ACC CCT TGG CTC GGG      294
                                        Met Thr Pro Trp Leu Gly
                                                        -20

CTC ATC GTG CTC CTG GGC AGC TGG AGC CTG GGG GAC TGG GGC GCC GAG      342
Leu Ile Val Leu Leu Gly Ser Trp Ser Leu Gly Asp Trp Gly Ala Glu
        -15                 -10                 -5

GCG TGC ACA TGC TCG CCC AGC CAC CCC CAG GAC GCC TTC TGC AAC TCC      390
Ala Cys Thr Cys Ser Pro Ser His Pro Gln Asp Ala Phe Cys Asn Ser
     1               5                  10                  15

GAC ATC GTG ATC CGG GCC AAG GTG GTG GGG AAG AAG CTG GTA AAG GAG      438
Asp Ile Val Ile Arg Ala Lys Val Val Gly Lys Lys Leu Val Lys Glu
                20                  25                  30

GGG CCC TTC GGC ACG CTG GTC TAC ACC ATC AAG CAG ATG AAG ATG TAC      486
Gly Pro Phe Gly Thr Leu Val Tyr Thr Ile Lys Gln Met Lys Met Tyr
            35                  40                  45

CGA GGC TTC ACC AAG ATG CCC CAT GTG CAG TAC ATC CAC ACG GAA GCT      534
Arg Gly Phe Thr Lys Met Pro His Val Gln Tyr Ile His Thr Glu Ala
        50                  55                  60

TCC GAG AGT CTC TGT GGC CTT AAG CTG GAG GTC AAC AAG TAC CAG TAC      582
Ser Glu Ser Leu Cys Gly Leu Lys Leu Glu Val Asn Lys Tyr Gln Tyr
     65                  70                  75

CTG CTG ACA GGT CGC GTC TAT GAT GGC AAG ATG TAC ACG GGG CTG TGC      630
Leu Leu Thr Gly Arg Val Tyr Asp Gly Lys Met Tyr Thr Gly Leu Cys
 80                  85                  90                  95

AAC TTC GTG GAG AGG TGG GAC CAG CTC ACC CTC TCC CAG CGC AAG GGG      678
Asn Phe Val Glu Arg Trp Asp Gln Leu Thr Leu Ser Gln Arg Lys Gly
                100                 105                 110

CTG AAC TAT CGG TAT CAC CTG GGT TGT AAC TGC AAG ATC AAG TCC TGC      726
Leu Asn Tyr Arg Tyr His Leu Gly Cys Asn Cys Lys Ile Lys Ser Cys
            115                 120                 125

TAC TAC CTG CCT TGC TTT GTG ACT TCC AAG AAC GAG TGT CTC TGG ACC      774
Tyr Tyr Leu Pro Cys Phe Val Thr Ser Lys Asn Glu Cys Leu Trp Thr
        130                 135                 140

GAC ATG CTC TCC AAT TTC GGT TAC CCT GGC TAC CAG TCC AAA CAC TAC      822
Asp Met Leu Ser Asn Phe Gly Tyr Pro Gly Tyr Gln Ser Lys His Tyr
    145                 150                 155

GCC TGC ATC CGG CAG AAG GGC GGC TAC TGC AGC TGG TAC CGA GGA TGG      870
Ala Cys Ile Arg Gln Lys Gly Gly Tyr Cys Ser Trp Tyr Arg Gly Trp
160                 165                 170                 175

GCC CCC CCG GAT AAA AGC ATC ATC AAT GCC ACA GAC CCC TGA GCGCCAGAC   921
Ala Pro Pro Asp Lys Ser Ile Ile Asn Ala Thr Asp Pro ***
                180                 185

CCTGCCCCAC CTCACTTCCC TCCCTTCCCG CTGAGCTTCC CTTGGACACT AACTCTTCCC   981
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AGATGATGAC AATGAAATTA GTGCCTGTTT TCTTGCAAAT TTAGCACTTG GAACATTTAA 1041

AGAAAGGTCT ATGCTGTCAT ATGGGGTTTA TTGGGAACTA TCCTCCTGGC CCCACCCTGC 1101

CCCTTCTTTT TGGTTTTGAC ATCATTCATT TCCACCTGGG AATTTCTGGT GCCATGCCAG 1161

AAAGAATGAG GAACCTGTAT TCCTCTTCTT CGTGATAATA TAATCTCTAT TTTTTTAGGA 1221

AAACAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1281

AAAA 1285

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Chemically Synthesized Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGCTCGAAAT TAACCCTCAC TAAAG 25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Chemically Synthesized Oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TACATCCATA CGGAAGTTTC CGAGAGT 27

CLAIMS

1. A recombinant DNA molecule encoding TIMP-3.

2. An expression vector comprising the DNA of claim 1.

3. A host cell transformed with the expression vector of claim 2.

4. A method for production of TIMP-3, said method comprising the steps:

a) culturing the host cells of claim 3 under conditions appropriate for expression of TIMP-3 and,

b) recovering said TIMP-3 from the cell culture.

5. An antisense DNA molecule of Claim 1.

6. A purified polypeptide comprising amino acid sequence encoded by the nucleotide sequence of the sequence listing.

7. An antibody to the purified polypeptide of Claim 6.

8. A method of treating a disease comprising administering to an individual suffering from that disease a pharmaceutical composition comprising TIMP-3 in a pharmaceutically acceptable excipient.

9. A method of claim 8 wherein the disease is cancer.

10. A method of claim 8 wherein the disease is arthritis.

11. A method of claim 8 wherein the disease is periodontal disease.

12. A method of claim 8 wherein the disease is osteoporosis.

13. A method of claim 8 wherein the disease is corneal ulceration.

14. A diagnostic test using a polynucleotide probe with sequence corresponding to all or part of the recombinant DNA molecule of Claim 1 to assess inflammatory conditions.

15. A diagnostic test using the antibody of Claim 7 to assess inflammatory conditions.

Amino Acid Content Graph [hTIMP-1 mature (Clark)]

Amino Acid		Count	Mol%	(%) 20 40 60 80 100
Ala	A	11	5.98	
Arg	R	10	5.43	
Asn	N	4	2.17	
Asp	D	5	2.72	
Cys	C	12	6.52	
Gln	Q	12	6.52	
Glu	E	9	4.89	
Gly	G	12	6.52	
His	H	6	3.26	
Ile	I	7	3.80	
Leu	L	18	9.78	
Lys	K	8	4.35	
Met	M	3	1.63	
Phe	F	10	5.43	
Pro	P	10	5.43	
Ser	S	13	7.07	
Thr	T	16	8.70	
Trp	W	3	1.63	
Tyr	Y	6	3.26	
Val	V	9	4.89	
Asx	B	0	0.00	
Glx	Z	0	0.00	
***	*	0	0.00	
Xxx	X	0	0.00	

FIGURE 1

Amino Acid Content Graph [hTIMP-2 mature (Langley)]

Amino Acid		Count	Mol%	(%) 20 40 60 80 100
Ala	A	13	6.70	
Arg	R	6	3.09	
Asn	N	6	3.09	
Asp	D	13	6.70	
Cys	C	12	6.19	
Gln	Q	8	4.12	
Glu	E	12	6.19	
Gly	G	13	6.70	
His	H	4	2.06	
Ile	I	17	8.76	
Leu	L	7	3.61	
Lys	K	18	9.28	
Met	M	5	2.58	
Phe	F	7	3.61	
Pro	P	12	6.19	
Ser	S	13	6.70	
Thr	T	7	3.61	
Trp	W	4	2.06	
Tyr	Y	7	3.61	
Val	V	10	5.15	
Asx	B	0	0.00	
Glx	Z	0	0.00	
***	*	0	0.00	
Xxx	X	0	0.00	

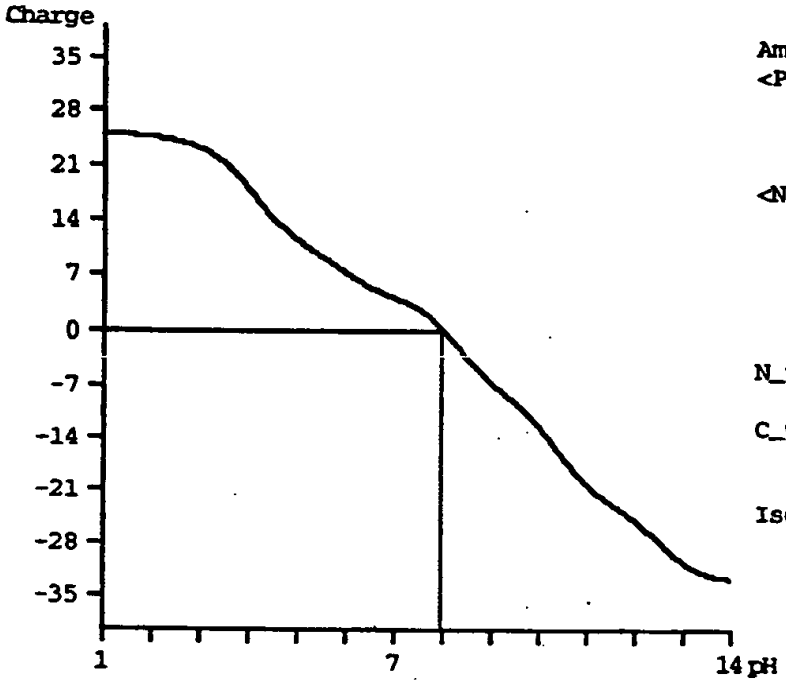
FIGURE 2

Amino Acid Content Graph [hTIMP-3 Mature]

Amino Acid		Count	Mol%	(%) 20 40 60 80 100
Ala	A	6	3.19	
Arg	R	8	4.26	
Asn	N	8	4.26	
Asp	D	7	3.72	
Cys	C	12	6.38	
Gln	Q	8	4.26	
Glu	E	6	3.19	
Gly	G	15	7.98	
His	H	5	2.66	
Ile	I	8	4.26	
Leu	L	15	7.98	
Lys	K	17	9.04	
Met	M	5	2.66	
Phe	F	6	3.19	
Pro	P	9	4.79	
Ser	S	12	6.38	
Thr	T	11	5.85	
Trp	W	4	2.13	
Tyr	Y	16	8.51	
Val	V	10	5.32	
Asx	B	0	0.00	
Glx	Z	0	0.00	
***	*	0	0.00	
Xxx	X	0	0.00	

FIGURE 3

Isoelectric Point [hTIMP-1 mature (Clark)]

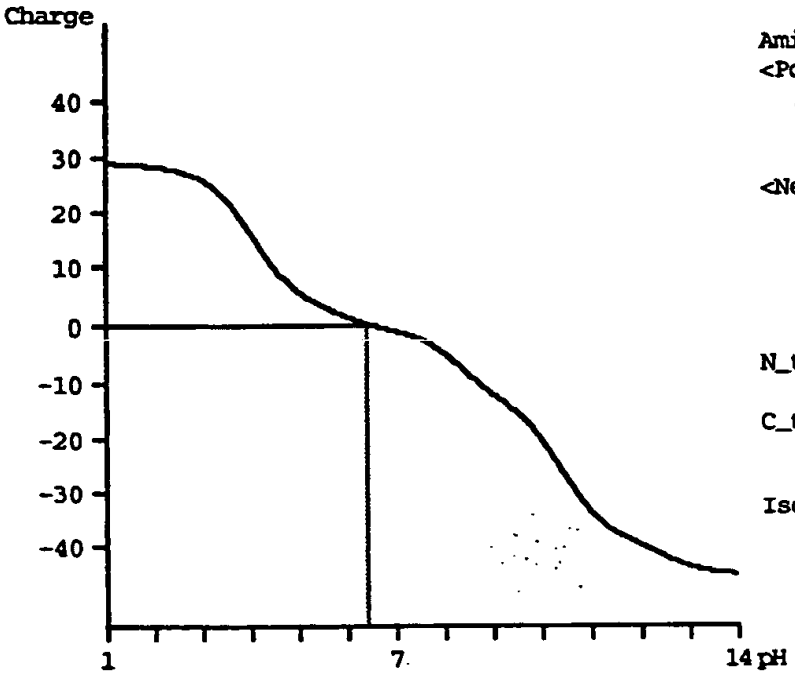


Amino Acid	Number	pKa
<Positive>		
Arg(R)	10	12.5
His(H)	6	6.0
Lys(K)	8	10.5
<Negative>		
Asp(D)	5	3.9
Cys(C)	12	8.3
Glu(E)	9	4.3
Tyr(Y)	6	10.1
N_terminal	Cys(C)	10.8
C_terminal	Ala(A)	2.4

Isoelectric point [pI] 8.00

FIGURE 4

Isoelectric Point [hTIMP-2 mature (Langley)]

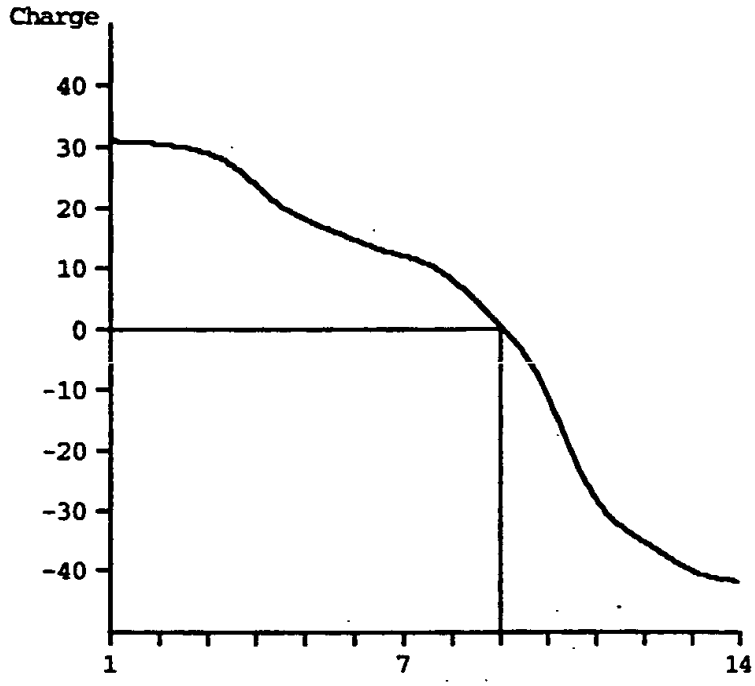


Amino Acid	Number	pKa
<Positive>		
Arg(R)	6	12.5
His(H)	4	6.0
Lys(K)	18	10.5
<Negative>		
Asp(D)	13	3.9
Cys(C)	12	8.3
Glu(E)	12	4.3
Tyr(Y)	7	10.1
N_terminal	Cys(C)	10.8
C_terminal	Pro(P)	2.0

Isoelectric point [pI] 6.45

FIGURE 5

Isoelectric Point [hTIMP-3 Mature]



Amino Acid	Number	pKa
<Positive>		
Arg(R)	8	12.5
His(H)	5	6.0
Lys(K)	17	10.5
<Negative>		
Asp(D)	7	3.9
Cys(C)	12	8.3
Glu(E)	6	4.3
Tyr(Y)	16	10.1
N_terminal	Cys(C)	10.8
C_terminal	Pro(P)	2.0

Isoelectric point [pI] 9.04

FIGURE 6

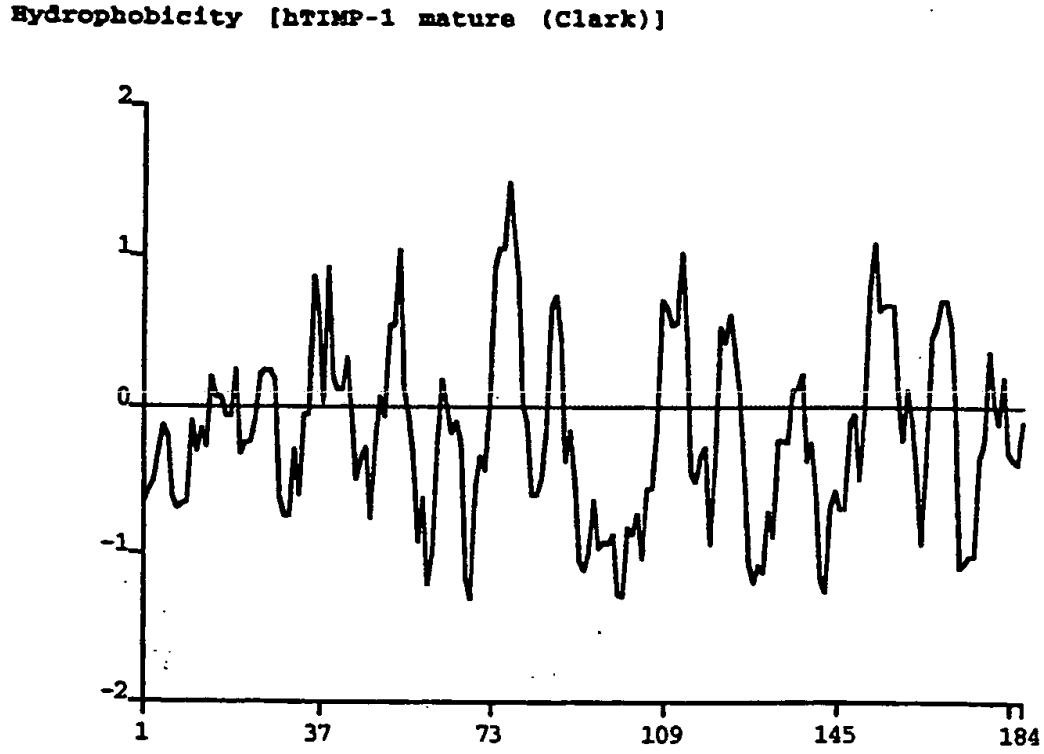

Hydrophobicity [hTIMP-1 mature (Clark)]
2
1
0
-1
-2
1
37
73
109
145
184

FIGURE 7

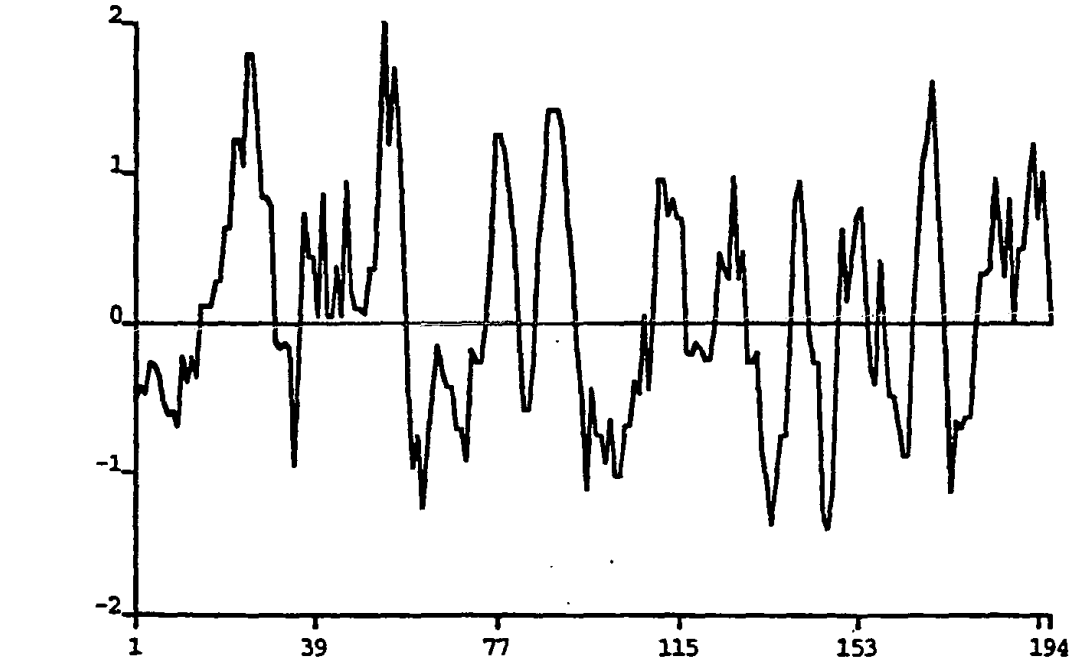

Hydrophobicity [hTIMP-2 mature (Langley)]
2
1
0
-1
-2
1
39
77
115
153
194

FIGURE 8

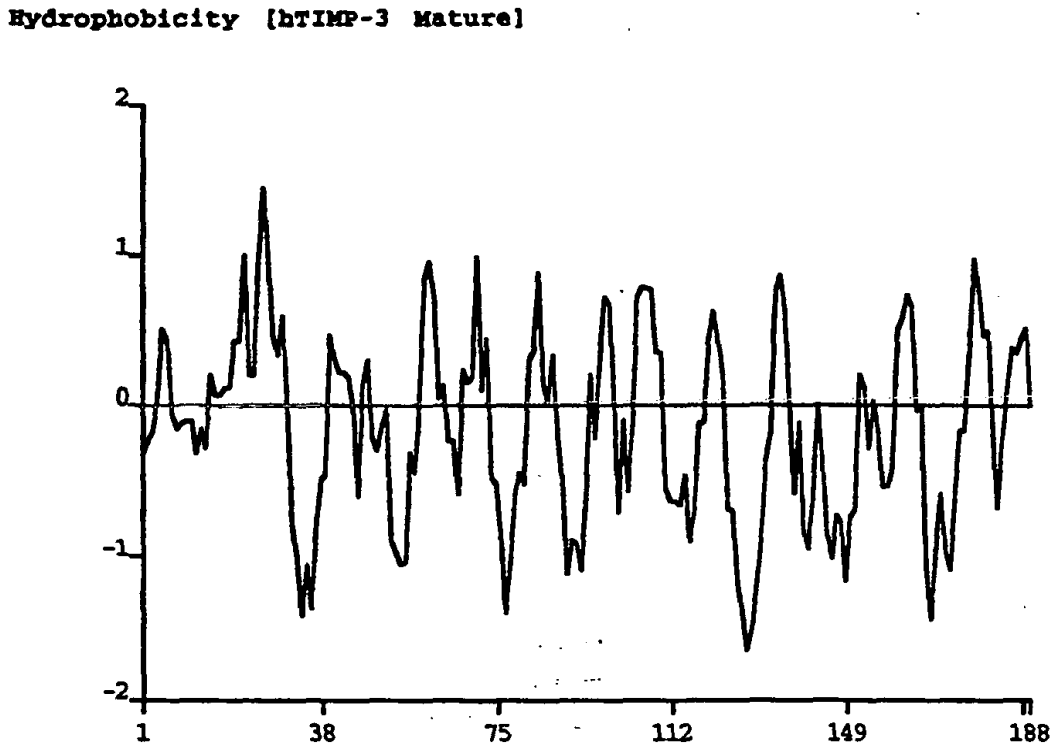


FIGURE 9

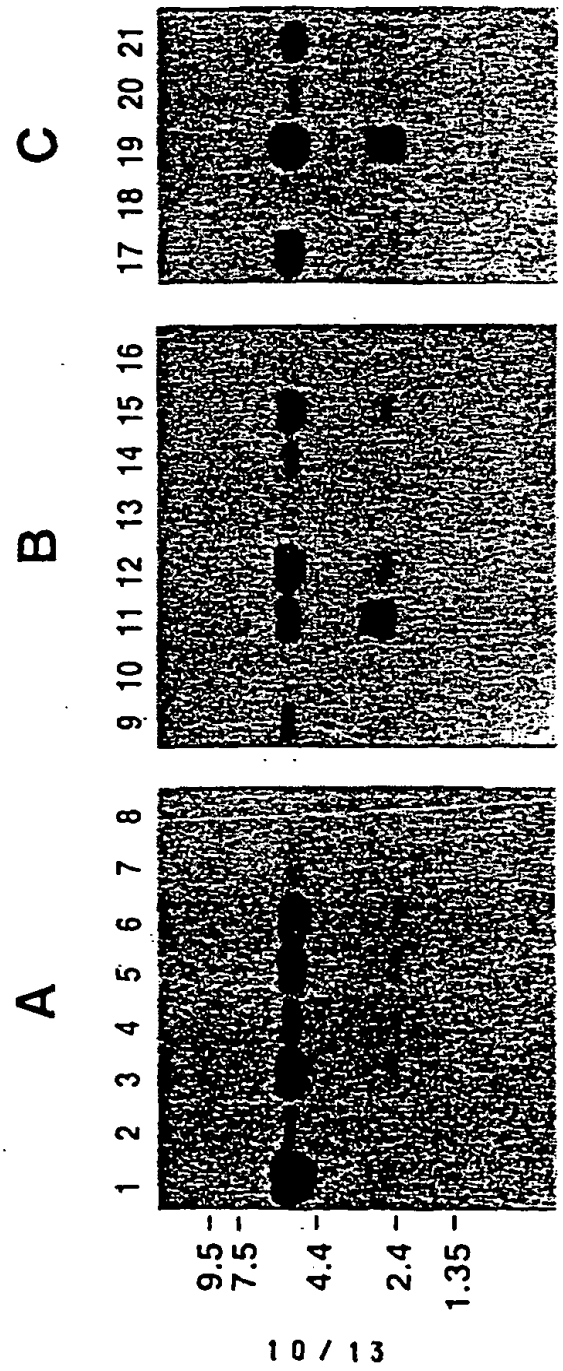


FIGURE 10

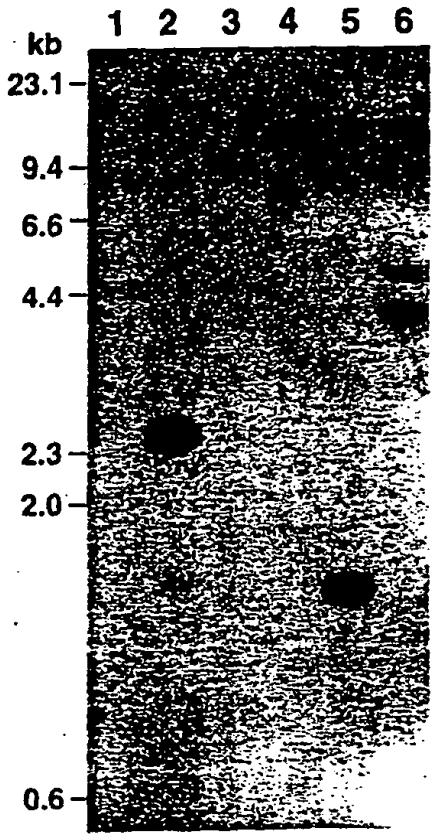
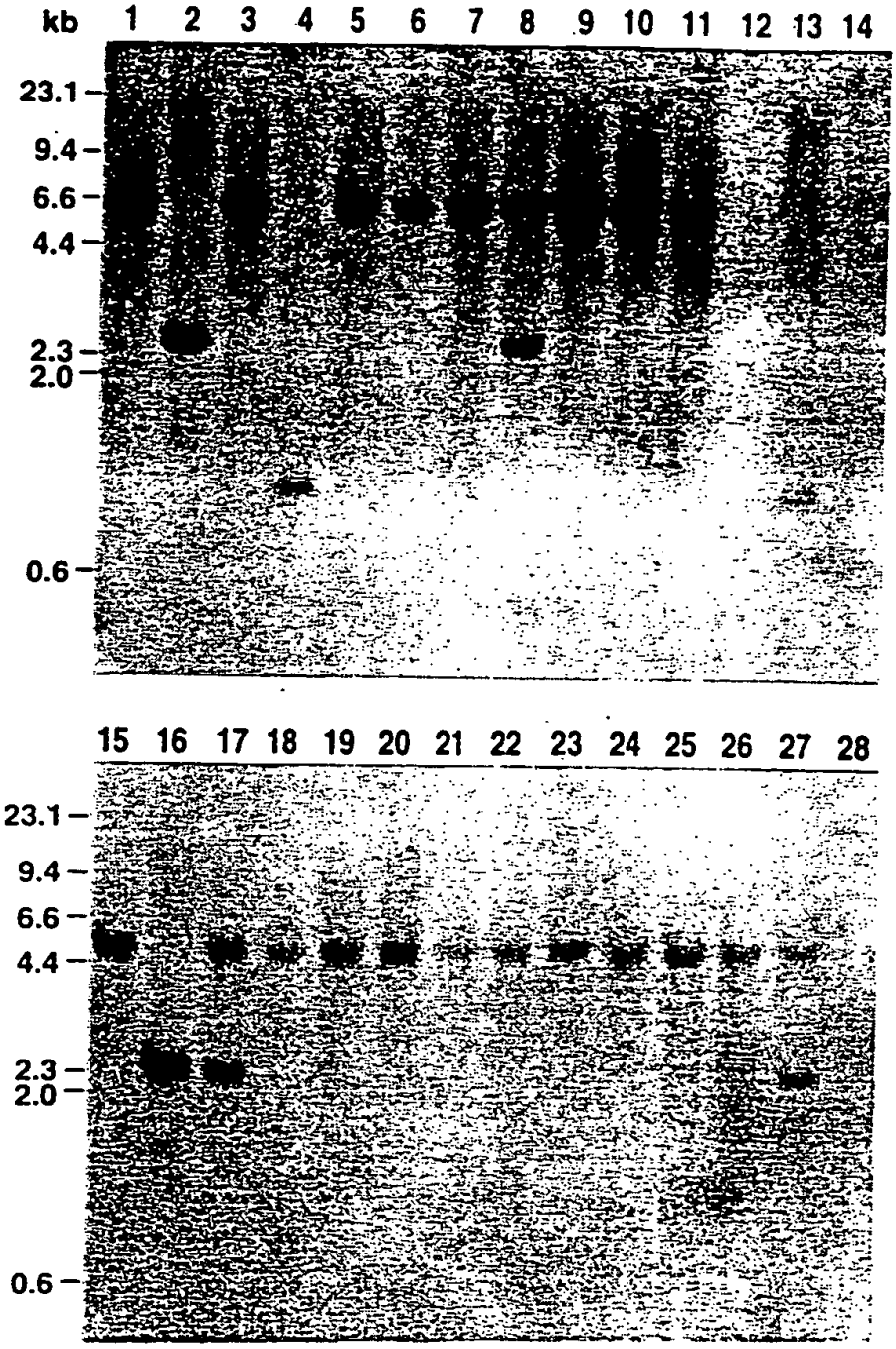


FIGURE 11


kb
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23.1
9.4
6.6
4.4
2.3
2.0
0.6
15 16 17 18 19 20 21 22 23 24 25 26 27 28
23.1
9.4
6.6
4.4
2.3
2.0
0.6

FIGURE 12

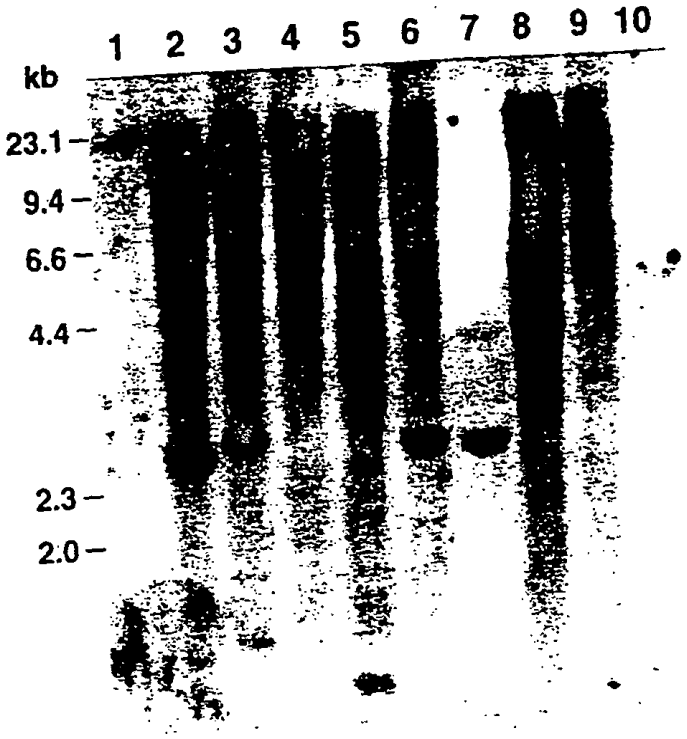

FIGURE 13